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Smokeless tobacco induced increases in hepatic lipid peroxidation, DNA damage and excretion of urinary lipid metabolites

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Summary. The possible role of reactive oxygen species in the toxicity of smokeless tobacco (ST) was explored. The effects of an aqueous smokeless tobacco extract (STE) at doses of 125, 250 and 500 mg STE/kg in rats on the induction of hepatic mitochondrial and microsomal lipid peroxidation and the incidence of hepatic nuclear DNA damage 24 hours post treatment were examined. Dose-dependent increases of 1.8, 2.3 and 4.4-fold in mitochondrial and 1.5, 2.1 and 3.6-fold in microsomal lipid peroxidation occurred at 125, 250 and 500 mg STE/kg, respectively, relative to control values. At these same three doses of STE, 1.3, 1.4 and 2.7-fold increases in hepatic DNA single-strand breaks occurred relative to control values. STE administration also resulted in significant increases in excretion of urinary metabolites. Urinary excretion of the four lipid metabolites malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON) was monitored by HPLC for 72 hours after treatment of rats with 125 and 250 mg STE/kg. Increases occurred in the excretion of the four lipid metabolites at every dose and time point with maximum increases in the excretion of all lipid metabolites being observed between 12 and 24 hours post treatment. The results suggest the involvement of an oxidative stress in the toxicity of STE.

Keywords: smokeless tobacco, lipid peroxidation, DNA single strand breaks, rats, urinary excretion, malondialdehyde, formaldehyde, acetaldehyde, acetone

Approximately one-third of all cancers in the United States are believed to be tobacco related (USPHS 1990). The use of smokeless tobacco products as snuff is damaging to both oral and general health and is associated with cancers of the lip, mouth, nasal cavities, oesophagus and gut (Brinton *et al.* 1984; Fielding 1985).

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The mechanism by which smokeless tobacco constituents produce genetic damage and cause tissue damage is not known. Although the precise site and mechanism of action of cytotoxicity of smokeless tobacco are not known, recent studies in our laboratories have shown that activation occurs when macrophages are incubated with an aqueous extract of smokeless tobacco with the resultant production of reactive oxygen species (Stohs *et*

al. 1993). Two other potential sources of reactive oxygen species in response to xenobiotics include hepatic mitochondria and microsomes (Bagchi M. *et al.* 1993).

In the present study we have examined the effects of an aqueous smokeless tobacco extract (STE) at three different doses on hepatic lipid peroxidation and formation of DNA single-strand breaks. Lipid peroxidation reflects the interaction between molecular oxygen and polyunsaturated fatty acids, resulting in the oxidative deterioration of the latter with the breakdown products including alcohols, aldehydes, ketones and ethers (Bus & Gibson 1979; Slater 1984). When lipid peroxides and peroxidation products accumulate, they leak from the organ or tissue into the blood stream and may be excreted in the urine (Yagi 1987). The detection of lipid peroxidation products in the urine provides a convenient and non-invasive method of assessing lipid peroxidation and oxidative stress (Shara *et al.* 1992). Therefore the four lipid metabolites malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON) were identified and quantitated in the urine of rats by high pressure liquid chromatography (HPLC) after treatment with STE, providing information on the ability of STE to induce an oxidative stress and tissue damage in these animals.

Materials and methods

Chemicals

Standardized smokeless tobacco (moist snuff) was purchased from the University of Kentucky tobacco and Health Research Institute, Lexington, KY. All other chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO), and were analytical grade or of the highest grade available.

Smokeless tobacco extract (STE)

Quantities of smokeless tobacco were mixed with 5 volumes (5 ml/g) of 0.10 M phosphate buffer, pH 7.0, and stirred at room temperature for 24 hours. The pH of the extracts was readjusted to 7.0 after one hour of stirring, and the extracts were centrifuged at 40 000 ***g*** for one hour. The supernatant fractions were filtered through a Millipore filter (0.45 μm), lyophilized and stored at −80°C. The extracts were reconstituted in phosphate buffer.

Animals and treatment

Female Sprague–Dawley rats weighing 160–180 g were purchased from Sasco, Inc. (Omaha, NE). All animals were housed two per cage and allowed to acclimatize to the environment for 4 or 5 days prior to experimental use. All animals were allowed free access to tap water and food (Purina Lab Chow). The rats were maintained at a temperature of 21°C with lighting from 0600 to 1800 h daily. Groups of rats were given single oral doses of 125, 250 or 500 mg STE/kg in phosphate buffer in a volume not exceeding 1 ml per animal. Control animals received the vehicle.

Preparation of mitochondria and microsomes

Following decapitation of the rats, livers were quickly removed and placed in ice-cold 50 mM Tris HCl buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol (Bagchi & Stohs 1993). The livers were homogenized with 5 ml buffer/g of tissue in a Potter Elvehjem homogenizer (Wheaton, NJ) fitted with a Teflon pestle using four 30-second strokes. Subcellular fractionation was achieved by differential centrifugation as described by Casals *et al.* (1985). Isolated mitochondrial and microsomal fractions were further purified by resuspension and centrifugation. Protein content of mitochondria and microsomes was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Lipid peroxidation

Lipid peroxidation was determined on hepatic mitochondria and microsomes from control and treated animals as previously described (Bagchi *et al.* 1992), based on the formation of thiobarbituric acid reactive substances (TBARS). Malondialdehyde was used as the standard and prepared according to the method of Largilliere and Melancon (1988). A molar extinction coefficient of 1.56×10^5/M/cm was used.

DNA single-strand breaks

DNA damage in hepatic nuclei from treated and control rats was measured as single-strand breaks by alkaline elution method (Wahba *et al.* 1989; White *et al.* 1981). DNA content was measured microfluorimetrically with 3,5-diaminobenzoic acid dihydrochloride as the complexing agent, with activation and emission wavelengths of 436 and 521 nm, respectively. The elution constant (k), which is used as a measure of DNA damage, was calculated from the formula $k = -2.30 \times$ slope of the plot of percentage DNA remaining on the filter versus volume of eluate.

Quantitation of urinary lipid metabolites

Rats were placed in metabolism cages (Nalgene Co., Rochester, NY) for urine collection. The animals were allowed free access to tap water but received no food during urine collection to avoid possible contamination of the urine with food particles. The urine collecting vessels were positioned over Styrofoam containers filled with dry ice in order to collect the urine in the frozen state over a 4.5-hour period, with the midpoints of collection times occurring at 0, 12, 24, 48 and 72 hours after STE administration. The detection of lipid peroxidation products in the urine provides a non-invasive method of assessing lipid metabolism and oxidative stress (Shara *et al.* 1992). High pressure liquid chromatography (HPLC) used in conjunction with gas chromatography–mass spectrometry (GC-MS) has provided conclusive identification and quantitation of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON) excretion in the urine of rats (Shara *et al.* 1992). The 2,4-dinitrophenylhydrazine derivatives of the four metabolic products of urine were quantitated by HPLC on a Waters μ-Bondapak C_{18} column, eluting with acetonitrile–water (51:49 v/v) mobile phase and using a UV absorbance detector at 330 nm as previously described (Shara *et al.* 1992).

Statistical methods

Significance between pairs of mean values was determined by Student's *t*-test. A $P < 0.05$ was considered significant for all analyses. Each value is derived from 4–6 animals.

Results

The ability of an aqueous smokeless tobacco extract (STE) to induce lipid peroxidation in hepatic mitochondria and microsomes, and hepatic DNA single-strand breaks as well as enhanced excretion of urinary metabolites, was investigated. The dose-dependent effects of the orally administered STE on lipid peroxidation in hepatic mitochondrial and microsomal membranes are presented in Table 1. Dose-dependent increases of 1.8, 2.3, and 4.4-fold in mitochondrial lipid peroxidation and 1.5, 2.1 and 3.6-fold in microsomal lipid peroxidation occurred at 125, 250 and 500 mg STE/kg, respectively, 24 hours post treatment relative to control values.

The cytotoxicity of STE was further examined by determining the incidence of hepatic DNA single-strand breaks (DNA damage) 24 hours after the administration of 125, 250 and 500 mg STE/kg to Sprague–Dawley rats. DNA single-strand breaks were determined by the alkaline method and are presented in Table 1. A dose-dependent increase in the incidence of DNA damage was observed, with increases of 1.3, 1.4 and 2.7-fold occurring at the three respective doses.

Determination of the urinary excretion of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON) by high pressure liquid chromatography (HPLC) constitutes a non-invasive method for assessing cytotoxicity and oxidative tissue damage by foreign chemicals. Therefore, groups of rats were treated orally with single, acute doses of 125 and 250 mg STE/kg in phosphate buffer, and the urinary excretion of these four lipid metabolites in nmol/kg body weight/4.5 hours was determined, with the midpoints of urine collection occurring at 0, 12, 24, 48 and 72 hours post treatment. Control animals received the vehicle. Increases occurred in the excretion of the four lipid metabolites at every dose and time point, with maximum increases for all metabolites being observed at approximately 12–24 hours post treatment. The results are presented in Figure 1.

Twelve hours after treatment, malondialdehyde (MDA) excretion increased by approximately 44% and by 96% after the administration of 125 and 250 mg STE/kg, respectively, relative to control values (Figure 1a). Twenty-four hours after the administration of these same two doses of STE, the urinary excretion of MDA increased by approximately 55 and 121%, respectively, as compared to the control values. In both cases, the urinary excretion of MDA decreased after the 24-hour timepoint.

Twelve hours post treatment, the urinary excretion of formaldehyde (FA) increased by approximately 37 and 105% relative to control values after administration of 125 and 250 mg STE/kg, respectively (Figure 1b). Twenty-four hours after administration of these same two doses of STE, the urinary excretion of FA increased approximately 49 and 100%, respectively, relative to control values. The excretion of FA decreased thereafter.

The dose and time-dependent effects of STE on the urinary excretion of acetaldehyde (ACT) are presented in Figure 1c. Oral administration of 125 and 250 mg STE/kg resulted in increases in ACT excretion of approximately 42 and 91%, respectively, relative to control values 12 hours post treatment. Twenty-four hours post-treatment, ACT excretion increased by approximately 44 and 110%, respectively, at these same two doses. The excretion of ACT decreased after the 24-hour timepoint.

Smokeless tobacco extract (mg/kg)	Lipid peroxidation (nmol MDA/mg protein)		DNA single-strand breaks elution constants ($\times 10^{-3}$)
	Mitochondria	Microsomes	
0	1.95 ± 0.28	2.69 ± 0.32	4.88 ± 0.81
125	3.12 ± 0.36*	4.09 ± 0.58*	6.72 ± 1.02*
250	4.58 ± 0.59*	5.67 ± 0.36*	6.93 ± 0.33*
500	8.62 ± 0.48*	9.82 ± 0.54*	12.97 ± 1.28*

Table 1. Hepatic lipid peroxidation and DNA damage in Sprague–Dawley rats 24 hours after treatment with smokeless tobacco extract

Female Sprague–Dawley rats received 125, 250 or 500 mg of an aqueous smokeless tobacco extract (STE) orally in buffer as a single dose. Control animals received the vehicle. The animals were killed 24 hours after treatment. Each value is the mean ± s.d. of 4–6 animals $^*P < 0.05$ with respect to the respective control group.

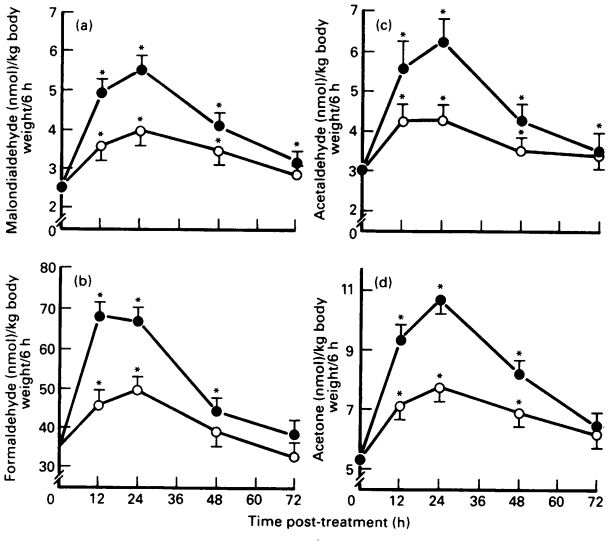

Figure 1. Sprague–Dawley rats were treated orally with a single dose of a STE, and urine samples were collected for 4.5 hours over dry ice with the midpoints of collection occurring at 0, 12, 24, 48 and 72 hours. The data represent the excretion of: a, malondialdehyde (MDA); b, formaldehyde (FA); c, acetaldehyde (ACT); and d, acetone (ACON). Control values did not differ significantly from the 0-hour time point over the 72 hours of the study. $^*P < 0.05$ with respect to the control group. Dose of STE: ●, 250; ○, 125 mg/kg.

The urinary excretion of acetone (ACON) increased by approximately 40 and 80% 12 hours after the administration of 125 and 250 mg STE/kg, respectively, while 24 hours post treatment, the urinary excretion increased by approximately 52 and 107%, respectively, relative to control values (Figure 1d). The excretion of ACON decreased after the 24-hour timepoint. The results clearly demonstrate that STE produces both dose and time-dependent effects on the urinary excretion of these four lipid metabolites, and therefore produces oxidative tissue damage.

Discussion

Previous studies have demonstrated that aqueous and methanolic extracts of smokeless tobacco can inhibit collagen synthesis in osteoblast-like cells (Lenz *et al.* 1990). It has also been shown that a water soluble extract of snuff incubated *in vitro* with human lymphocytes results in an inhibition of lymphokine activated killer cell activity as well as DNA synthesis (Lindemann & Park 1988). In the present study, the dose-dependent effects of an aqueous smokeless tobacco extract (STE) on hepatic microsomal and mitochondrial lipid peroxidation were examined. Furthermore, the effects of STE on two other indices of tissue damage, namely DNA single-strand breaks in hepatic nuclei and increases in the production of the urinary lipid metabolites MDA, FA, ACT and ACON, were also assessed.

The data clearly demonstrate that STE induces dose dependent increases in hepatic mitochondrial and microsomal lipid peroxidation as well as hepatic DNA damage (Table 1). These results strongly suggest that the STE induces an oxidative stress which may involve the production of reactive oxygen species. The administration of STE also resulted in dose-dependent increases in the urinary excretion of the lipid metabolites formaldehyde (FA), malondialdehyde (MDA), acetaldehyde (ACT) and acetone (ACON) (Figure 1a–d). Peroxidation of lipids is known to result in the production of a wide variety of aldehydes, dialdehydes, alcohols, ketones, alkanes, alkenes and other related products (Bus & Gibson 1979; Slater 1984).

The increased excretion of MDA, FA, ACT and ACON at 24 hours after administration of STE correlates well with the increases in hepatic lipid peroxidation. Furthermore, a good correlation exists between the peroxidation of membrane lipids and the increase in DNA single-strand breaks. The in-vitro incubation of STE with macrophages, hepatic mitochondria and microsomes

results in enhanced chemiluminescence and increased lipid peroxidation (Stohs *et al.* 1993), indicative of the production of reactive oxygen species. Thus, the constituents in STE may directly interact with macrophages as well as hepatic mitochondria and microsomes to initiate production of reactive oxygen species. The increased excretion of the four lipid metabolites is indicative of oxidative tissue damage and has been shown to be associated with the oxidative stress produced by a wide variety of xenobiotics including paraquat, menadione, endrin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), carbon tetrachloride and adriamycin (Bagchi D. *et al.* 1992; 1993; 1994; Shara *et al.* 1992).

The effects of STE *in vivo* in rats on the production of nitric oxide by isolated peritoneal exudate (macrophage) cells and when incubated with cultured J774A.1 macrophage cells has recently been examined (Hassoun *et al.* 1994). Nitric oxide is a highly reactive endogenous chemical produced by activated macrophages which serves as a mediator for expressing cytotoxic activity. STE when administered to rats produced a significant increase in the production of nitric oxide by isolated peritoneal exudate cells. Similar results were observed when macrophage cells in culture were incubated with STE. When the antioxidant vitamin E succinate was administered to rats prior to treatment with STE, a marked decrease in nitric oxide production occurred in the isolated peritoneal macrophage cells. Similar results were observed when the J774A.1 macrophages were cultured in the presence of vitamin E succinate and STE. Taken together, these results indicate that STE activates macrophages with the resultant production of reactive oxygen species (Hassoun *et al.* 1994).

The mechanisms involved in the formation of MDA, FA, ACT and ACON in response to toxicants as STE which induce an oxidative stress are not entirely clear. The enhanced urinary excretion of these metabolites may occur via a variety of mechanisms, including enhanced lipid peroxidation and oxidative stress induced cell injury, and/or the enhanced β-oxidation of fatty acids (Foster 1987; Schulz 1991). The mechanisms involved in the formation of MDA by lipid peroxidation are well known (Slater 1984).

The enhanced formation of ACON in response to disease states, such as diabetes, as a consequence of increased β-oxidation, is well established (Foster 1987). However, increased lipid peroxidation also plays a role in increased ACON. For example, the herbicide paraquat is known to stimulate reactive oxygen species mediated lipid peroxidation, with the greatest increase in the excretion of the four lipid metabolites being observed with ACON following paraquat administration to rats (Bagchi D. *et al.* 1993).

Winters *et al.* (1988) demonstrated that rat liver microsomes metabolize glycerol to FA. Glycerol is a product of the metabolism of triglycerides by adipose tissue and other tissues that possess the enzyme glycerol kinase, which activates glycerol. Liver and brown adipose tissues are known to have high glycerol kinase levels (Winters *et al.* 1988). Recent studies by Clejan and Cederbaum (1993) have shown that paraquat stimulates microsomal and cytochrome P-450-dependent oxidation of glycerol to FA. Stimulation of the microsomal oxidation of glycerol to FA may be a mechanism which is common to many xenobiotics which induce an oxidative stress.

Dhanakoti and Draper (1987) have examined the disposition of radiolabelled MDA that was administered to rats. MDA appears to be extensively metabolized to acetate and carbon dioxide. Recent studies in our laboratories have shown that the administration of MDA to rats results in a large increase in the urinary excretion of ACT (Akubue *et al.* 1994). Thus, based on these observations, ACT excretion in the urine may arise in part as an intermediate product in the breakdown of MDA which is formed by lipid peroxidation. Other possible sources of FA might include the breakdown of MDA to ACT or acetate and a one-carbon fragment, and/or the cleavage of a one-carbon fragment from acetoacetatic acid with the formation of ACON.

In summary, the results support the hypothesis that an aqueous extract of smokeless tobacco (STE) induces the production of reactive oxygen species. Taken together with previous studies, the results indicate that STE may act at multiple sites. The reactive oxygen species which are formed may lead to enhanced lipid peroxidation (Bagchi M. *et al.* 1993) as well as other tissue damaging effects such as DNA single-strand breaks, contributing to the cytotoxicity of STE.

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